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# A fundamental system of cellular energy homeostasis regulated by PGC-1 $\alpha$

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**Maintenance of ATP levels is a critical feature of all cells. Mitochondria are responsible for most ATP synthesis in eukaryotes. We show here that mammalian cells respond to a partial chemical uncoupling of mitochondrial oxidative phosphorylation with a decrease in ATP levels, which recovers over several hours to control levels. This recovery occurs through an increased expression of the transcriptional coactivator peroxisome proliferator-activated receptor-coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) and mitochondrial genes. Cells and animals lacking PGC-1 $\alpha$  lose this compensatory mechanism and cannot defend their ATP levels or increase mitochondrial gene expression in response to reduced oxidative phosphorylation. The induction of PGC-1 $\alpha$  and its mitochondrial target genes is triggered by a burst of intracellular calcium, which causes an increase in cAMP-response-element-binding protein and transducer of regulated cAMP-response-element-binding proteins actions on the PGC-1 $\alpha$  promoter. These data illustrate a fundamental transcriptional cycle that provides homeostatic control of cellular ATP. In light of this compensatory system that limits the toxicity of mild uncoupling, the use of chemical uncoupling of mitochondria as a means of treating obesity should be re-evaluated.**

mitochondria | uncoupling

**M**itochondria are responsible for producing most of the ATP needed for energy-requiring reactions in eukaryotic cells (1). In the process of oxidative phosphorylation (OXPHOS), a proton gradient across the inner mitochondrial membrane is coupled to the synthesis of ATP by the F<sub>1</sub>F<sub>0</sub>ATPase at complex V. Mitochondrial metabolism is dynamic and can be modulated in response to external stimuli. For example, the  $\beta$ -adrenergic pathway is activated upon cold exposure in mice, which causes brown adipose tissue to shift toward more uncoupled respiration (2–4). In uncoupled respiration, the protons pumped into the intermembrane space leak back into the mitochondrial matrix, bypassing ATP production and generating heat.

Mitochondria themselves can elicit intracellular signaling pathways that alter nuclear gene expression, mitochondria number, and function (5, 6). This signaling process is referred to as retrograde signaling. Retrograde signaling has been well studied in the budding yeast, *Saccharomyces cerevisiae*. Yeast lacking mtDNA show increased expression of many genes important for the function of mitochondria, such as citrate synthase, and several proteins have been identified in yeast that regulate retrograde signaling (7). Little is known about the regulatory proteins involved in the mechanisms of retrograde signaling in higher eukaryotes. Similarly, how and to what extent higher eukaryotic cells can defend their ATP levels during chronic challenges has not been extensively explored.

Peroxisome proliferator-activated receptor-coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) was first identified as a cold-inducible transcriptional coactivator of peroxisome proliferator-activated receptor  $\gamma$  that can activate a program of adaptive thermogenesis (8). PGC-1 $\alpha$  has since been shown to be a dominant regulator of mitochondrial function, biogenesis, and respiration in many tissues (9–13). Ectopic expression of PGC-1 $\alpha$  in white adipocytes increases cellular respiration and genes important for mitochondrial function, such as uncoupling protein 1, cytochrome *c* (Cyt *c*), and Cyt *c* oxidase subunit II (COX II). Furthermore, increased expression of PGC-1 $\alpha$

increases mitochondrial volume density and cristae density (14). PGC-1 $\alpha$  also can activate a program of fiber-type switching in skeletal muscle, including increased mitochondrial content and the expression of myofibrillar proteins characteristic of type 1 and type 2a muscle fibers (15). PGC-1 $\beta$ , the closest homolog of PGC-1 $\alpha$ , has also been shown to regulate mitochondrial biogenesis and respiration in cells and transgenic animals (13, 16, 17).

2,4-Dinitrophenol (DNP), a chemical mitochondrial uncoupler, was widely used in the 1930s as a treatment for obesity (18–20). Mild doses were amazingly effective, often causing rapid loss of adipose mass and body weight. While unregulated use led to reports of toxicity and even death, it was not as lethal as might have been expected for a compound that disrupted OXPHOS. This tolerance indicates that cells can sustain mild chemical uncoupling, perhaps by using an energy compensatory mechanism. In the present study, we have investigated whether uncoupling of the mitochondrial membrane potential could activate a compensatory pathway controlling ATP homeostasis. Indeed, chemical uncoupling increases the expression of PGC-1 $\alpha$  and PGC-1 $\beta$ , as well as the expression of several target mitochondrial genes. Studies using PGC-1 $\alpha$  null cells and animals indicate that PGC-1 $\alpha$  is required for the induction of mitochondrial gene expression, recovery of ATP levels, and cell survival. Together, these data illustrate a fundamental system of energy homeostasis whereby cells and tissues use PGC-1 $\alpha$  to recover energy balance and also demonstrate that a powerful compensatory system exists that limits the toxicity of mild uncoupling.

## Results

**Chemical Uncoupling Causes a Transient Drop in ATP Levels.** To examine whether and how mammalian cells might adapt to the chemical uncoupling of mitochondrial OXPHOS, we measured ATP levels and cell death in fibroblasts treated with the chemical mitochondrial uncoupler carbonyl cyanide *p*-(trifluoromethoxy) phenyl hydrazone (FCCP) (21). Perhaps surprisingly, fibroblasts treated with up to 200  $\mu$ M FCCP for 16 h remained 98% viable (Fig. 1A), although viability decreased to  $\approx$ 80% in the cells treated with 200  $\mu$ M FCCP for 24 h. Treatment of fibroblasts with FCCP caused a 25% in ATP levels at 30 min of treatment (Fig. 1B), but this drop

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The authors declare no conflict of interest.

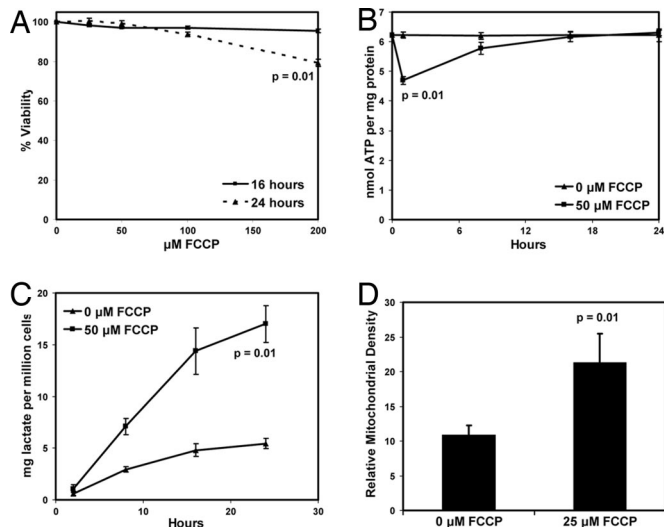
Abbreviations: PGC-1, peroxisome proliferator-activated receptor-coactivator 1; OXPHOS, oxidative phosphorylation; CREB, cAMP-response-element-binding protein; ACREB, dominant-negative CREB; TORC, transducer of regulated CREB protein; Cyt *c*, cytochrome *c*; COX II, Cyt *c* oxidase subunit II; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenyl hydrazone; NRF, nuclear respiratory factor; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate; AMPK, AMP-activated protein kinase; TFAM, mitochondrial transcription factor A.

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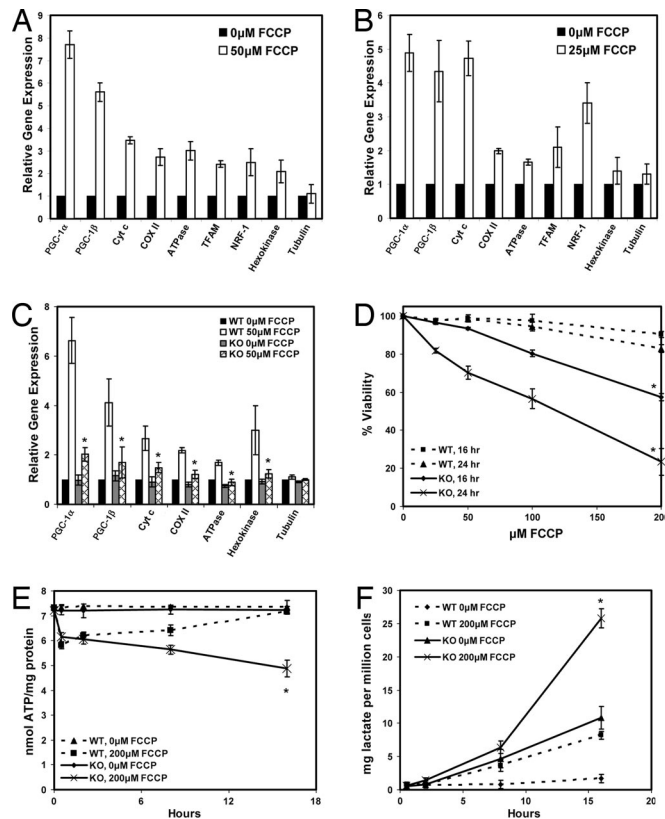


**Fig. 1.** Chemical uncoupling induces multiple metabolic changes. (A) Cells were treated with 0, 25, 50, 100, or 200  $\mu\text{M}$  FCCP for 16 or 24 h, and viability was measured by using the LDH assay ( $P = 0.01$  compared with untreated cells at 24 h). (B) ATP levels were measured after fibroblasts were treated with 0 or 50  $\mu\text{M}$  FCCP for 0, 2, 8, 16, or 24 h ( $P = 0.01$  compared with the untreated cells at 0.5 h). (C) Lactate was measured after fibroblasts were treated with 0 or 50  $\mu\text{M}$  FCCP for 2, 8, 16, or 24 h ( $P = 0.01$  compared with the untreated cells). (D) Mitochondrial volume density in cells treated with 0 and 25  $\mu\text{M}$  FCCP for 72 h ( $P = 0.01$ ).

was transient, as ATP levels later recovered to those of the untreated cells. We also examined the amount of lactate produced during this experiment. Over 24 h, cells treated with FCCP secreted four times more lactate than the untreated cells (Fig. 1C). This finding suggests that glycolytic metabolism helps cells withstand a decrease in OXPHOS caused by chemical uncoupling.

We asked whether this mitochondrial uncoupling had any affect on mitochondrial density by using electron microscopy to quantify the volume of mitochondria in cells treated with FCCP. Fibroblasts were exposed to 0 or 25  $\mu$ M FCCP for 72 h, and mitochondrial volume density was measured as described (14, 22). Fibroblasts treated with FCCP displayed a 1.8-fold higher mitochondrial volume than control cells and higher levels of Cyt *c* protein [Fig. 1D and [supporting information \(SI\) Fig. 7](#)], suggesting that chronic uncoupling induces a compensatory program that not only helps to recover ATP levels, but alters mitochondrial density well.

**Partial Chemical Uncoupling of Mitochondria Induces PGC-1 $\alpha$ , PGC-1 $\beta$ , and Mitochondrial OXPHOS Genes.** To determine whether changes in the expression of genes related to mitochondrial function accompany the metabolic alterations seen in cells treated with FCCP, we measured the mRNAs encoding the PGC-1 coactivators, powerful regulators of mitochondrial gene expression, and expression of genes of the electron transport system. Using real-time PCR, we observed an induction of mRNAs encoding for Cyt *c*, COX II, F<sub>1</sub> $\beta$ -ATPase, and nuclear respiratory factor 1 (NRF-1). In addition, PGC-1 $\alpha$  and PGC-1 $\beta$  mRNA increased 7.5- and 5.5-fold, respectively, upon treatment with FCCP for 16 h (Fig. 2A). In extended treatment with FCCP for 72 h, mRNA for PGC-1 $\alpha$  and PGC-1 $\beta$  was elevated 4-fold, and the mRNAs encoding Cyt *c*, COX II, F<sub>1</sub> $\beta$ -ATPase, and NRF-1 were also significantly elevated relative to vehicle-treated cells (Fig. 2B). To determine whether a different mitochondrial uncoupler could also increase these genes, fibroblasts were treated with DNP. Sixteen hours of treatment with DNP increased PGC-1 $\alpha$  and PGC-1 $\beta$  mRNA  $\approx$ 7-fold; mRNAs for Cyt *c*, COX II, F<sub>1</sub> $\beta$ -ATPase, and hexokinase were similarly induced (SI Fig. 8). These data indicate that chemical uncoupling of the

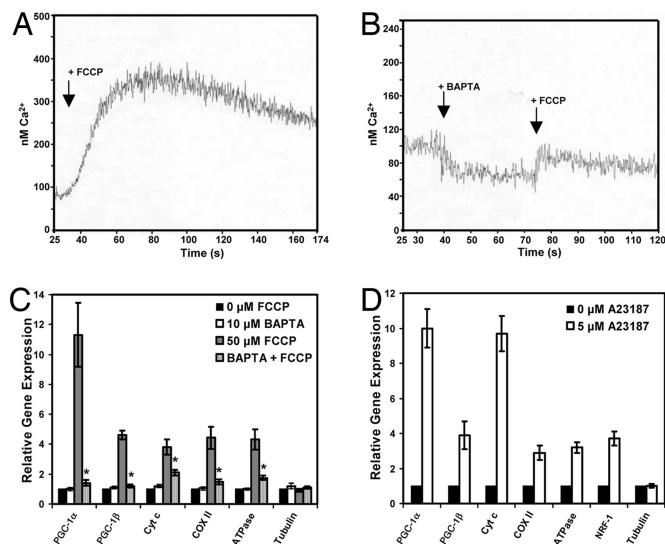


**Fig. 2.** Chemical uncoupling induces OXPHOS genes and the PGC-1 coactivators. (A) Fibroblasts were treated with 0 or 50  $\mu$ M FCCP for 16 h. RNA was measured by using real-time PCR (all points are significant except for tubulin  $P < 0.01$ ). (B) The experiment in A was repeated, but cells were treated for 72 h with 0 or 25  $\mu$ M FCCP (all points are significant except for tubulin and hexokinase,  $P < 0.01$ ). (C) WT and PGC-1 $\alpha^{-/-}$  preadipocytes (KO) were treated with 0 or 50  $\mu$ M FCCP for 16 h, and mRNAs were measured. The PGC-1 $\alpha$  measurement represents exon 2, which is still present in the KO cells ( $P < 0.05$  compared with the WT FCCP-treated cells). (D) WT and PGC-1 $\alpha$  KO preadipocytes were treated with 0, 25, 50, 100, and 200  $\mu$ M FCCP for 16 or 24 h. Viability was measured by using the LDH cytotoxicity assay ( $P < 0.01$  compared with the WT FCCP-treated cells). (E) ATP levels in the WT and PGC-1 $\alpha$  KO cells were measured after treatment with 50  $\mu$ M FCCP ( $P = 0.02$  compared with the WT FCCP-untreated cells at 16 h). (F) Lactate levels in the media of WT and KO cells treated with 200  $\mu$ M FCCP for the were measured ( $P = 0.005$  compared with the WT FCCP-treated cells at 16 h).

mitochondrial electron transport chain induces genes of mitochondrial OXPHOS and mRNA encoding the PGC-1 coactivators.

**PGC-1 $\alpha$  Is Required for the Induction of Mitochondrial Gene Expression, Recovery of ATP Levels, and Cell Survival.** We investigated whether the induction of PGC-1 $\alpha$  is required for the uncoupling-mediated induction of the compensatory metabolic program. Immortalized preadipocytes isolated from the brown fat of WT and PGC-1 $\alpha^{-/-}$  mice (23) were treated with FCCP for 16 h. Similar to what was seen in fibroblasts, this treatment induced a significant increase in the expression of mRNAs encoding PGC-1 $\alpha$ , PGC-1 $\beta$ , and several genes of the electron transport system (Fig. 2C). Strikingly, cells lacking PGC-1 $\alpha$  had an almost complete ablation of the induction of PGC-1 $\beta$  and OXPHOS genes, which indicates that PGC-1 $\alpha$  is almost absolutely required for the induction of the mitochondrial electron transport genes induced by partial mitochondrial uncoupling.

To determine whether PGC-1 $\alpha$  contributes to cell survival during reduced mitochondrial function, a cytotoxicity assay was used to examine cell viability after treatment with FCCP in both the



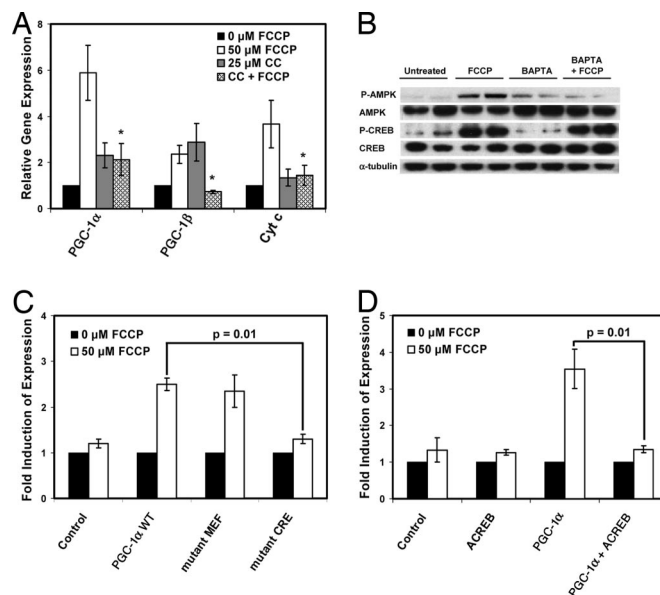
**Fig. 3.** The induction of PGC-1 $\alpha$ , PGC-1 $\beta$ , and OXPHOS genes requires increased [Ca<sup>2+</sup>]<sub>i</sub> levels. (A) Fibroblasts were loaded with Fura-2, and [Ca<sup>2+</sup>]<sub>i</sub> levels were measured (34, 54) as 50  $\mu$ M FCCP was added. (B) [Ca<sup>2+</sup>]<sub>i</sub> levels were measured as in A first with the addition 10  $\mu$ M BAPTA and the subsequent addition of FCCP. (C) Fibroblasts were pretreated for 1 h with 0 or 10  $\mu$ M BAPTA followed by 16 h of treatment with 0 or 50  $\mu$ M FCCP, and mRNAs were measured ( $P < 0.01$  compared with the FCCP-treated cells). (D) Fibroblasts were treated with 0 or 5  $\mu$ M A23187 for 16 h (all points are significant except for tubulin,  $P < 0.01$ ), and mRNAs were measured.

WT and PGC-1 $\alpha$  null cells. Whereas the WT cells showed very little death with up to 200  $\mu$ M FCCP at either time point, the PGC-1 $\alpha$  null cells showed a very substantial loss of viability (Fig. 2D). Only 80% and 60% of the mutant cells survived 100 and 200  $\mu$ M FCCP, respectively, after 16 h. After 24 h, only 30% of the mutant cells survived 200  $\mu$ M FCCP compared with 85% of control cells. Thus, PGC-1 $\alpha$  contributes substantially to the ability of these cells to survive partial uncoupling of the electron transport chain.

We next studied whether PGC-1 $\alpha$  plays a role in the recovery of the ATP levels seen after treatment with FCCP (Fig. 1B). Both WT and PGC-1 $\alpha^{-/-}$  cells displayed an initial 20% decrease in ATP levels (Fig. 2E). After 16 h, the WT cells completely recovered their ATP levels to those of the untreated cells, whereas the PGC-1 $\alpha^{-/-}$  cells could not restore their ATP levels. By 16 h, the ATP levels in the mutant cells were  $\approx$ 68% of the ATP levels in the untreated cells. In the same experiments, we measured lactate levels produced. After 16 h, the FCCP-treated WT cells secreted approximately three times more lactate than the untreated WT cells (Fig. 2F). The untreated PGC-1 $\alpha^{-/-}$  cells secreted significantly more lactate than both the treated and untreated WT cells, which was also true of the FCCP-treated PGC-1 $\alpha^{-/-}$  cells. These experiments together indicate that the recovery of ATP levels and cell survival seen after chronic treatment with FCCP requires PGC-1 $\alpha$ .

### Intracellular Calcium Plays a Critical Role in the PGC-1 $\alpha$ Pathway.

Mitochondria store significant quantities of calcium, and mitochondrial uncoupling is known to cause an increase in intracellular calcium ( $[Ca^{2+}]_i$ ) levels (6, 24–28). These data are of particular interest because the induction of PGC-1 $\alpha$  in other contexts has been shown to be regulated by components of the calcium signaling pathways (17, 29). 1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (BAPTA), a membrane soluble calcium chelator (30), has been shown to block the rise in  $[Ca^{2+}]_i$  caused by the uncoupling agent FCCP (24, 31–33). Using the calcium-sensitive dye Fura-2 (34), we observed a 3-fold increase in  $[Ca^{2+}]_i$  when fibroblasts were treated with FCCP (Fig. 3A). Pretreatment of cells with BAPTA



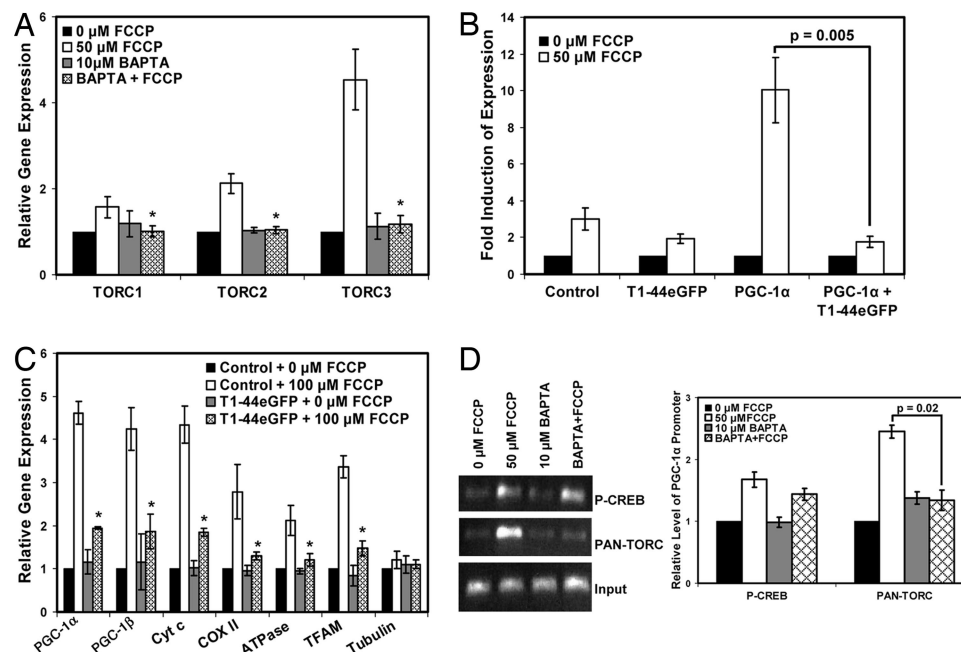
**Fig. 4.** AMPK and CREB are involved in the mechanism inducing PGC-1 $\alpha$ . (A) Fibroblasts were pretreated for 1 h with 0 or 25  $\mu$ M compound C (CC) followed by 16 h of treatment with 0 or 50  $\mu$ M FCCP. mRNAs were measured ( $P < 0.02$  compared with FCCP-treated cells). (B) Fibroblasts were treated as in Fig. 3C. Western blots were used to measure protein levels. (C) PGC-1 $\alpha$  promoter constructs with a luciferase reporter containing mutations in the MEF or CRE site were transfected into fibroblasts (PGC-1 $\alpha$  WT is the unmutated PGC-1 $\alpha$  2-kb promoter), then treated with FCCP. Luciferase units were normalized to  $\beta$ -galactosidase and to the cells treated with 0  $\mu$ M FCCP ( $P = 0.01$ ). (D) ACREB was cotransfected with the PGC-1 $\alpha$  2-kb promoter into fibroblasts. The cells were treated with FCCP, and luciferase units were measured and normalized as in C ( $P = 0.01$ ).

largely suppressed this rise in  $[Ca^{2+}]_i$  (Fig. 3B). To determine whether reducing the FCCP-mediated  $[Ca^{2+}]_i$  rise with BAPTA could block the expression of PGC-1 $\alpha$  and the other OXPHOS genes, fibroblasts were pretreated with BAPTA before treatment with FCCP. Indeed, the rise in PGC-1 $\alpha$  mRNA caused by FCCP was completely blocked (Fig. 3C); the increases in the mRNAs for Cyt c, COX II, and F<sub>1</sub> $\beta$ -ATPase caused by FCCP were also ablated.

To test whether compounds that increased  $[Ca^{2+}]_i$  levels had the same effect on PGC-1 $\alpha$  and mitochondrial gene expression as mitochondrial uncouplers, we treated fibroblasts for 16 h with the calcium ionophore A23187. As seen after treatment with FCCP, the mRNAs for PGC-1 $\alpha$ , PGC-1 $\beta$ , and the mRNAs for Cyt *c*, COX II, F<sub>1</sub> $\beta$ -ATPase, and NRF-1 all were increased (Fig. 3D). These data strongly suggest that altered calcium flux is a key component in the mechanism of this mitochondrial retrograde pathway regulated by PGC-1 $\alpha$ .

AMP-activated protein kinase (AMPK) is activated during low energy conditions, particularly when the ratio of AMP/ATP increases (35). AMPK activation is also known to induce PGC-1 $\alpha$  gene expression in muscle (36, 37). As expected, AMPK is activated upon treatment with FCCP (Fig. 4B) (33, 38). To investigate the role of AMPK in the uncoupling-induced homeostatic pathway, we inhibited AMPK and looked at the subsequent induction of PGC-1 $\alpha$  and OXPHOS gene expression. Fibroblasts were pretreated with the AMPK inhibitor compound C for 1 h, followed by treatment with FCCP. Whereas FCCP alone induced PGC-1 $\alpha$ , PGC-1 $\beta$ , and Cyt *c* mRNA levels, the addition of compound C almost completely blocked the further induction of those genes (Fig. 4A). However, pretreatment of cells with BAPTA blocks the activation of AMPK (Fig. 4B), suggesting that calcium signaling is upstream of the activation of AMPK by FCCP.





**Fig. 5.** TORC plays a critical role in the uncoupling-mediated induction of PGC-1 $\alpha$  and mitochondrial genes. (A) Fibroblasts were treated as described in Fig. 3C ( $P < 0.01$  compared with the FCCP-treated samples), and mRNAs for TORC1, TORC2, and TORC3 were measured. (B) Fibroblasts were transfected with the PGC-1 $\alpha$  2-kb promoter, cotransfected with T1-44eGFP, and then treated with FCCP. Luciferase units were normalized to the cells treated with 0  $\mu$ M FCCP ( $P = 0.005$ ). (C) Preadipocytes constitutively expressing T1-44eGFP or the eGFP control were treated with either 0 or 50  $\mu$ M FCCP for 16 h, and mRNAs were measured ( $P < 0.01$  compared with FCCP-treated control cells). (D) ChIP was used to measure phospho-CREB protein and TORC protein bound to the PGC-1 $\alpha$  promoter upon treatment with FCCP. Fibroblasts were treated as described in Fig. 3C. Cells were cross-linked, and protein-DNA complexes were harvested by using phospho-CREB or PAN-TORC antibodies. The amount of PGC-1 $\alpha$  promoter was then quantified by using PCR and real-time PCR with PGC-1 $\alpha$ -specific primers ( $P = 0.02$ ).

**Involvement of cAMP-Response-Element-Binding Protein (CREB) in the Induction of the PGC-1 $\alpha$  Promoter.** To elucidate the mechanism of this energy cycle further, we investigated the cis- and trans-acting factors that play a role in the induction of the PGC-1 $\alpha$  gene upon chemical uncoupling. Activity of a promoter segment containing 2 kb of the 5' flanking region of the PGC-1 $\alpha$  promoter driving a luciferase gene (29) was induced 3-fold upon treatment with FCCP (Fig. 4C). Transfections of the PGC-1 $\alpha$  promoter harboring mutations in the MEF2 and CREB binding sites were analyzed. Promoter constructs with a mutation in the MEF2 binding site showed no difference in activity compared with the WT PGC-1 $\alpha$  promoter (Fig. 4C). However, mutation of the CREB binding site (39) resulted in an almost complete ablation of the increase in the PGC-1 $\alpha$  promoter activity in response to FCCP.

To investigate the role of the transcription factor CREB in the FCCP-mediated induction of the PGC-1 $\alpha$  promoter, we examined the induction of the WT 2-kb PGC-1 $\alpha$  promoter in the presence of a dominant-negative version of the CREB protein (ACREB) (40). ACREB, a fusion of an acidic amphipathic domain and the leucine zipper dimerization domain of CREB, heterodimerizes with endogenous CREB and prevents it from binding to DNA. ACREB completely blocked the induction of the PGC-1 $\alpha$  promoter observed upon treatment with 50  $\mu$ M FCCP (Fig. 4D). Typically CREB protein requires activation by phosphorylation, often at Ser-133. Fig. 4B shows that treatment with FCCP increases the regulatory Ser-133 phosphorylation of CREB. However, BAPTA, which blocks the induction of PGC-1 $\alpha$  mRNA and OXPHOS gene expression, does not block the phosphorylation of CREB. These data together suggest a required role for CREB in the control of the PGC-1 $\alpha$  promoter, but also suggest other regulated steps in this pathway must exist.

**Involvement of Transducer of Regulated CREB Proteins (TORCs) in the Regulation of PGC-1 $\alpha$ .** We sought to identify other factors in the calcium signaling pathway that were sensitive to BAPTA. Recently,

the TORC coactivators have emerged as important regulators of CREB-dependent transcription, irrespective of the phosphorylation state of CREB (41–48). Given this and their involvement in calcium signaling pathways (43, 44), the TORCs seemed to be plausible regulators in the pathway by which uncoupling induces PGC-1 $\alpha$ . To determine whether TORCs are involved in the PGC-1 $\alpha$  energy cycle, fibroblasts were treated with FCCP. TORC1 mRNA increased 1.6-fold, TORC2 mRNA increased 2.1-fold, and TORC3 mRNA increased 4.2-fold (Fig. 5A); pretreatment with BAPTA completely blocked the induction of all three TORC mRNAs. To determine whether TORCs play a role in the FCCP-mediated induction of the PGC-1 $\alpha$  promoter, we measured the induction of the WT 2-kb PGC-1 $\alpha$  promoter in the presence of a dominant-negative version of TORC (43). The dominant-negative TORC protein (T1-44eGFP) is a fusion protein of the conserved CREB binding domain of TORC1 and eGFP. T1-44eGFP has been shown to block the activation of CREB by TORC1, TORC2, and TORC3 (43). In the absence of T1-44eGFP, the PGC-1 $\alpha$  promoter was induced 9.5-fold upon treatment with FCCP (Fig. 5B). However, T1-44eGFP completely blocked the FCCP-mediated induction of the PGC-1 $\alpha$  promoter, suggesting strongly that TORCs are required for the induction of the PGC-1 $\alpha$  promoter under these conditions.

In a similar experiment, preadipocytes constitutively expressing T1-44eGFP were treated with FCCP for 16 h, and expression of several mitochondrial genes was subsequently measured. Control cells showed a 4.6-fold increase in PGC-1 $\alpha$  mRNA and increases in PGC-1 $\beta$ , Cyt c, COX II, F<sub>1</sub> $\beta$ -ATPase, and mitochondrial transcription factor A (TFAM) mRNAs (Fig. 5C). However, cells expressing T1-44eGFP had a blunted induction of mRNAs for all of these genes, again suggesting that TORCs are required for the PGC-1 $\alpha$  energy cycle induced by uncoupling.

ChIP was used to measure the association of TORCs with the endogenous PGC-1 $\alpha$  promoter in the presence of FCCP and



(San Diego, CA). Antibodies were from Cell Signaling (Danvers, MA) except for anti-Pan TORC (Calbiochem). The 2-kb PGC-1 $\alpha$  promoter and promoter mutants have been described (29). ACREB was provided by Charles Vinson (40) (National Institutes of Health, Bethesda, MD). The dominant-negative TORC (T1-44eGFP) and control (eGFP) were provided by Novartis (Basel, Switzerland) (43). Immortalized preadipocytes from PGC-1 $\alpha$  WT and null mice (23, 53) and preadipocytes expressing T1-44eGFP were provided by Marc Uldry (Dana-Farber Cancer Institute).

**Cell Culture, Transfection, and ChIP.** 10T 1/2 fibroblasts were grown in DMEM (10% FBS). Immortalized preadipocytes from PGC-1 $\alpha$  WT and  $-/-$  mice were cultured in DMEM (20% FBS). For the reporter gene assays, cells were transfected overnight with SuperFect (Qiagen, Valencia, CA) and treated with FCCP for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase (Promega, Madison, WI), and then compared with the empty pGL3basic vector. pGL3basic vector, pSV vector, and eGFP served as the controls for the PGC-1 $\alpha$  2-kb promoter, ACREB, and T1-44eGFP, respectively. ChIP was performed with a ChIP-IT kit (Active Motif, Carlsbad, CA).

**Analysis of Gene Expression.** RNA was isolated by using TRIzol (Invitrogen, Carlsbad, CA) and measured by using iSCRIPT and SYBRGreen (Bio-Rad, Hercules, CA). mRNA levels were normalized to actin mRNA, and then relative mRNA levels were determined by using the  $\Delta\Delta C_t$ .

**Electron Microscopy, Lactate, ATP, and Viability Measurements.** ATP levels were measured by using the ATP Determination Kit (Invitrogen). Lactate in the media was quantified with Lactate Reagent (Trinity Biotech, Bray, Ireland). Viability was measured by using the LDH cytotoxicity kit (Roche, Indianapolis, IN). Electron microscopy was performed, and mitochondrial volume density was measured as described (14, 22).

**Calcium Measurements.** Fura-2 ratios were used to calculate  $[Ca^{2+}]$  as described (34, 54). For the measurements, EGTA was added to chelate the extracellular calcium. FCCP was then added followed by digitonin and EGTA/Tris for calibration. When BAPTA was used, it was added before the addition of FCCP.

**Animal Experiments.** All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee. Mice (see *SI Text*) were injected with 50 mg/kg DNP (90% saline, 10% DMSO solution) or 0 mg/kg DNP (90% saline, 10% DMSO solution). Mice were killed after 5 h.

**Statistical Analysis.** Results are expressed as  $\pm$ SD. Two-tailed Student's *t* tests were used to determine *P* values.

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